Supplemental Protocols

Protocol S1.

TXTL reaction for CRISPR-based repression

This protocol will demonstrate how to test five different dSpyCas9 sgRNA targets, all targeting the plasmid P70a-deGFP, and one non-targeting control, in a TXTL reaction. This protocol can be generalized for TXTL reactions to test other CRISPR applications, and for other reaction volumes or component concentrations, with all components other than DNA being the same.

Materials

- 3 M potassium glutamate (Sigma-Aldrich G1149)
- 180 mM magnesium glutamate (Sigma-Aldrich 49605)
- 500 mM maltodextrin (Sigma-Aldrich 419672)
- 40% w/v PEG8000 (Sigma-Aldrich P2139)
- TXTL extract, 33 µl aliquot for 100 µl total reaction volume
- Energy Buffer
- Amino Acid mix
- Chi6 DNA (annealed oligos)
- DNA
    - dSpyCas9 plasmid (pCB453)
    - sgRNA DNA (linear, any of sg2-sg20 and sg-nt)
    - P70a-deGFP reporter plasmid
- 96 V-bottom well plate (Corning 3357)
- Sealing mat for 96 V-bottom well plate (Corning 3080)

TXTL reaction

1. Pre-incubate the 96 V-bottom well plate with sealing mat at 29°C for at least 30 minutes prior to the reactions being pipetted into the plate in step X.
2. Retrieve DNA (stored at -20°C) and make sgRNA DNA stocks for each of six sgRNA targets (including the non-target) at 10 nM in water. Make dSpyCas9 plasmid stock at 100 nM in water. Make P70a-deGFP plasmid stock at 50 nM in water.
3. Retrieve magnesium glutamate, potassium glutamate, and PEG (stored at 4°C). Prepare maltodextrin fresh by dissolving in water to 500 mM (using 360 g/mol).
4. Retrieve TXTL extract, amino acid mix, and energy buffer (stored at -80°C) and thaw on ice.
5. Add components directly to cell extract tube:

Volume [µL]	Ingredient	Final Concentration	Initial Concentration	Units
33	TXTL Extract			
15.57	Water	fill to 88% volume		
3	K-Glu	90	3000	mM
2.22	Mg-Glu	4	180	mM
6	maltodextrin	30	500	mM

5.56	energy buffer	1	18	X
17.65	amino acid mix	3	17	mM
2	chi6	2	100	µM
3	dSpyCas9 plasmid	3	100	nM

6. Vortex gently but thoroughly to ensure that chi6 is mixed well in the reaction.
7. The total reaction volume is now 88 µl (88% reaction volume). Pipette 10.56 µl into a 1.5 mL tube and add 1.44 µl water. This tube is the blank reaction for background subraction.
8. To the remaining 77.44 µl (still at 88% reaction volume) add the reporter plasmid:

Volume [µL]	Ingredient	Final Concentration	Initial Concentration	Units
1.76	P70a-deGFP	1	50	nM

9. Vortex gently but thoroughly and split the reaction (now 79.2 µl at 90% volume) into six tubes of 10.8 µl (90% of 12 µl).
10. Add 1.2 µl sgRNA DNA to each respective tube (one tube for each of the six different sgRNA, including the non-targeting). Reactions are now 12 µl and 100% volume.
11. Vortex gently. Pipette 5 µl into each of two wells of the 96 V-bottom plate for each reaction tube (for two technical replicates), including the blank reaction.
12. Measure GFP fluorescence kinetics at 29°C on a plate reader for 16 hours at three minute intervals.
13. Repeat entire protocol as necessary for replicates.

Tips and alternatives:

- Any components, including DNAs, that will be in all reactions (like dSpyCas9 in the given example protocol) can be added to the table in step 5. For example, a DNA encoding an anti-CRISPR protein could be added to the table.
- Pre-incubations can be used to express one gene for a certain time before adding another DNA. For example, one could add an sgRNA to the reaction in step 5, skip step 8 (do not add reporter plasmid yet), then add DNAs encoding anti-CRISPR genes in step 10 (instead of different sgRNA) and incubate for 2-3 hours at 29°C to express the encoded genes, then add the final reporter plasmid. Volumes and concentration may have to be adjusted from the protocol above.
- MYtxtl from the company MYcroarray can be used, then only DNAs and water need to be added.

Data Processing

The plate reader should export a spreadsheet, with time points in the first column, and fluorescence intensity value for each well of the microplate in subsequent columns.

1. For each time point, subtract the average fluorescence intensity of the blank reactions from all other reactions. This is the background subtraction.
2. Optional: To convert fluorescence intensity values to concentration of fluorescent protein, use a standard curve calibration. To make a standard curve calibration,

dilute pure recombinant GFP (Cell Biolabs STA-201) in PBS 1X (Sigma-Aldrich P5493) to concentrations of 0.1, 0.33, 1, 3.3, 10, 33 μM. Add 5 μl of each concentration of deGFP to the well plate and measure the fluorescence intensity. Fit the data to a line and extract the equation of the line. The calibration is only useful for the specific conditions used to make the calibration (including plate reader, well plate, reaction volume, optics position, PMT gain, excitation-emission wavelengths, lamp energy, etc.).

3. Take the average and standard error of measurement for all repeated reactions.
4. Time-course data can now be visualized. Make sure to always include controls. For example, plot the non-targeting sgRNA control with any targeting sgRNA run (like Figure 1B).
5. For endpoint fold-repression, divide the final non-targeting sgRNA fluorescence intensity value (at 16 hours) by that of the targeting sgRNA (like Figure 3A).

Protocol S2.

Using TXTL to identify PAMs recognized by a Type 2 Cas nuclease

Table of Contents

Materials Needed

- Two tubes of extract from the MyTXL *in vitro* cell-free protein expression kit (MYcroarray MYtxtl-70-24-M)
- 50 nM plasmid DNA containing a PAM library (see below for a protocol to construct the library)
- 20 nM linear DNA expressing gRNAs targeting the PAM library, the reporter plasmid, and a sequence not found in either plasmid (see below for how to order gBlocks expressing the gRNAs)
- 20-40 nM of either midiprepped plasmid DNA or linear DNA expressing the Cas protein of interest (see below for a discussion of the options for expressing the Cas protein).
- Oligos chi6.FWD and chi6.REV to block RecBCD degradation of linear DNA (see below for sequence)
- Primers CSMpr1308-1311 for cloning the PAM library (see below for sequence)
- Plasmid CSM160 for preparing the PAM library
- (Optional) Primers T7.FWD/REV for creating linear DNA expressing a Cas protein from genomic DNA or a vector without a bacterial promoter.
- (Optional) Midiprepped DNA of the reporter plasmid P70a-deGFP concentration should be 30.
- (If the Cas protein is expressed from a T7 promoter) Midiprepped plasmid of the P70a-T7Pol concentration should be 30 nM.
- (If the Cas protein is expressed from a pET vector) 50mM IPTG in nuclease free water
- NEB Q5 HotStart Polymerase (NEB: M0493S)
- NEBuilder HiFi DNA Assembly Cloning Kit (NEB: E5520S)
- NEB 5-alpha Electrocompetent Cells (High Efficiency) (NEB: C2987I)
- 10 mM dNTPs
- Oligos RL133/134 to add adapters to cleaved PAM library (see below for sequences)
- Nextera indexing primers. The sequences can be found here.

All plasmid DNA should be prepared using a midiprep kit (e.g. ZymoPure) and eluted in nuclease free water.

All linear DNA should be suspended in nuclease free water.

Assembling materials for the assay

Annealing the Chi6 oligos

Annealed Chi6 oligos are added to the TXTL reaction to retard degradation of linear DNA. Prepare a stock of 100μM of annealed Chi6 oligos (DOI 10.1002/bit.26333) by mixing 25μL of 100μM stocks of chi6.FWD and chi6.REV oligos in water and annealing them in a thermocycler with the following program:

95 ˚C for 5 minutes
reduce by 1˚C every 15s for 79 cycles
Hold at 10˚C

chi6.FWD	TCACTTCACTGCTGGTGGCCACTGCTGGTGGCCACTGCTGGTGGCCACTG CTGGTGGCCACTGCTG GTGGCCACTGCTGGTGGCCA
chi6.REV	TGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAGCAGTGGCCACCAG CAGTGGCCACCAGCA GTGGCCACCAGCAGTGAAGTGA

Annealed oligos can be stored at -20˚C for years.

Preparing the PAM library

We prepared the PAM library (https://benchling.com/s/seq-pndsuPeZr34iN6vq4gT1) in this manuscript using the Q5 site-directed mutagenesis kit as described in Leenay et al. 2016 (DOI 10.1016/j.molcel/2016.02.031). We have also created similar PAM libraries using Gibson Assembly. To create a PAM library using Gibson Assembly, we recommend using the plasmid CSM160; https://benchling.com/s/seq-eLacsHLas5p2Is8nyyCQ) as a backbone, and synthesizing the gBlock CSM-GB089 (a PAM library expressing 10 variable nucleotides with the appropriate spacer and binding site for NGS primers; https://benchling.com/s/seq-DUXeCAflnVOOhxcgBwR6) using IDT or a similar company. To assemble the library, order the following primers:

CSMpr1308	TTTTCTGTTCAGCTCACTCAAAGGCGGTAAaccaataaaaaacgcccggcggcaaccgag
CSMpr1309	TGGTGAATATGTCAAACATCCACTCTGCGGacggttatccacagaatcagggataacgc
CSMpr1310	gcgttatcccctgattctgtggataaccgtCCGCAGAGTGGATGTTTGACATATTCACCA
CSMpr1311	ctcggttgccgccgggcgtttttttattggtTTACCGCCTTTGAGTGAGCTGAACAGAAAA

Then set up two 50μL PCR reactions as follows using the Q5 enzyme.

Template	Primers	Annealing Temperature	Extension Time

CSM160	CSMpr1308 (F), CSMpr1309 (R)	72˚C	41 sec (1.64kB)
GB089	CSMpr1310 (F), CSMpr1311 (R)	72˚C	12 sec (0.48kB)

DpnI digest the backbone before purifying by adding 1µL of DpnI enzyme to the CSM160 PCR reaction and incubating at 37˚C for 1hr. PCR purify the backbones, then use the protocol from the NEB HiFi Assembly kit to assemble the pieces with a 1hr incubation at 50˚C. For a negative control, assemble with only the backbone.

PCR purify the two assembly reactions, eluting each in 6µL of elution buffer. Electroporate into NEB 5-alpha electrocompetent cells using the attached protocol.

After recovering the cells, use 20µL of the recovered cells to make a series of 1:10 dilutions in SOC media for both the positive and negative samples. Drop plate 10µL of the diluted cells onto LB+Chlor plates to estimate the number of transformants and incubate the plates overnight at 37˚C. Dilute the remaining cells 1:50 into LB+Chlor media and grow overnight at 37C.

In the morning, estimate the number of transformants and background using the drop plates. You should get ~1-2e6 transformants with a ~1:30,000 ratio of transformants to background. Note that CSM160 contains an mRFP gene that is replaced by the cloning. Uncleaved CSM160 can be visualized by looking for red colonies.

Make frozen stocks of the library as desired, then midi-prep the rest of the library using the ZymoPure Midiprep kit (Zymo D4200), elute the library in H20. The sequence of the resulting plasmid is here: https://benchling.com/s/seq-IcEy1Pvthfi9eCsFOdlA. The assembled 10N library can be available from the Beisel lab upon request.

Ordering DNA to express gRNA

1) Order gBlocks to express the crRNA of interest in TXTL. The gRNA should target either the positive or negative strand of the PAMSCANR spacer. An example gBlock that expresses the processed form of the FnCpf1 crRNA is here (CSM-GB099; https://benchling.com/s/seq-v9VSiOso6NUdZzmjiXpa), and an example sgRNA for Cas9 is here (https://benchling.com/s/seq-ewkCFmsNwsgIO7bxFJ1Q). Note that the gBlocks express the gRNA from a strong promoter. The spacer size may need to be adjusted depending on the Cas protein of interest.
2) Order a gBlock expressing the crRNA targeting a spacer not found in the P70a-deGFP plasmid or the PAM library. An example gBlock that expressed the processed form of the FnCpf1 crRNA targeting such a spacer is here (CSM-GB151; https://benchling.com/s/seq-OEsvWAWFOCXc9eo7ptSZ).
3) (Optional, but highly recommended if possible) If a PAM that is recognized by the Cas protein is already known, design a gBlock that targets a spacer flanked by that PAM on the P70a-deGFP reporter plasmid. An example gBlock expressing the processed form of the FnCpf1 crRNA targeting a TTTC sequence found in the P70a-deGFP reporter plasmid is here: (CSM-GB098; https://benchling.com/s/seq-XNZovPzyBddjIhT5feMZ).

Resuspend the lyophilized gBlocks in H20 to 20nM.

Preparing DNA to express the Cas nuclease

We have successfully expressed Cas nucleases in TXTL in three different ways.

Express the Cas protein from a plasmid under a constitutive promoter:
We have had success cloning Cas genes into the pBAD18 backbone under the control of constitutive promoters using the following protocol. First, clone the Cas protein into the pBAD18 backbone multiple cloning site. Second, replace the AraC gene and the ARA promoter with the constitutive J23108 promoter and a strong RBS. It is unclear why this sequence of steps is necessary, but it is.

Express the Cas protein from pET vector:
The Cas protein can cloned into a pET vector, a common vector used for protein structure studies. Use standard cloning techniques to insert the Cas protein of interest into the MCS.

Express the Cas protein from linear DNA:
We have found that some Cas proteins are very difficult (or impossible) to clone in *E coli*. To avoid this problem, linear DNA can be used to express the Cas proteins in TXTL. There are two options for generating linear DNA:

A. **Order the Cas protein as a gBlock.** Design a gBlock containing the codon-optimized version of your Cas protein. Ensure that the Cas protein is expressed from either a strong or medium constitutive promoter such as J23119 or J23108, or from the T7 promoter (An example gBlock used to express FnCpf1 is here: https://benchling.com/s/seq-YvEL8ir9XKqlsezI2EYZ). Resuspend the lyophilized gBlock expressing the Cas protein to 50nM in H20.

B. ***PCR the Cas protein from the prokaryotic genome or from a vector without a bacterial promoter.*** If genomic DNA containing the sequence of the Cas protein of interest, primers can be designed to add the T7 promoter, Shine-Delgarno sequence, and T7 terminator (Adapted from primer design for the NEB PureExpress kit LINK)

T7.FWD	GCGAATTAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAA AAA[5' binding site]
T7.REV	AAACCCCTCCGTTTAGAGAGGGGTTATGCTAGTTA[3' binding site]

Note that the first 3bp of the 5' binding site should be the start codon of the gene encoding the Cas protein, and the first 3bp of the 3' binding site should be the reverse complement of the stop codon of the gene encoding the Cas protein. PCR purify the PCR product and elute in H20.

Performing library cleavage in TXTL

Setting up a pilot cleavage assay

CRISPR-Cas RNPs assemble and cleave DNA at different rates when expressed in TXTL. Furthermore, we have found that some Cas nucleases are only active at particular temperatures. Therefore, if at all possible, run a control reaction in which the CRISPR-Cas RNP cleaves a reporter plasmid.

> *Note:* An alternative approach to this protocol is to clone the PAMSCANR spacer with the appropriate PAM outside of the P70a-deGFP coding sequence. This enables the same crRNA to be used for both the pilot and PAM assay, but requires an additional cloning step. We have had excellent results cloning such small sequences using the Q5 Site-directed mutagenesis kit from NEB.

Ensure that the gRNA that targets a known PAM and a non-targeting gRNA is diluted to the appropriate concentration in H20.

Ensure that the Cas protein DNA is diluted to an appropriate concentration in TXTL.

Thaw the TXTL extract and then add the following reagents on ice:

		Total Vol.		Vol % in MM	
		100		0.8	
Vol. µL	Ingredient	Final	Stock	Units	
75	MyTXTL Extract				
1	IPTG	0.5	50	mM	
1	P70a-deGFP	0.5	50	nM	
0.667	P70a-T7POL	0.2	30	nM	
2	Annealed Chi6 oligos	2	100	uM	
0.333	water				

Gently but thoroughly vortex the master mix.

Split the master mix into three 1.5 ml centrifuge tubes at room-temperature by adding 9.6 µL to each tube. Refreeze the rest of the master mix for later use if desired.

Add the following DNA to each sub-reaction:

Sub-reaction #	DNA1	DNA2
1	H20	Targeting gRNA

2	Cas DNA	Non-targeting gRNA
3	Cas DNA	Targeting gRNA

Gently but thoroughly vortex each sub reaction, then add 5µL of each reaction to the bottom of a V-bottom plate, seal with a cap mat, and monitor the fluorescence at 30˚C in a plate reader. The TXTL reactions should demonstrate cleavage of the reporter gene dependent on having both the Cas protein and the targeting gRNA. Note the time at which the reporter gene expression plateaus: this is the time that the cleavage of the DNA is complete.

Troubleshooting: If no cleavage (or cleavage that takes more than 3-4 hr is observed) try higher concentrations of the Cas protein DNA, and try different temperatures (e.g 37˚C).

Setting up a TXTL reaction to assay PAM recognition

Ensure that the gRNA that targets the PAMSCANR spacer and a non-targeting gRNA is diluted to the appropriate concentration in H20.

		Total Vol.		Vol % in MM	
		100		0.8	
Vol. µL	Ingredient	Final	Stock	Units	
75	MyTXTL Extract				
1	IPTG	0.5	50	mM	
1	PAM library	0.5	50	nM	
0.667	P70a-T7POL	0.2	30	nM	
2	Annealed Chi6 oligos	2	100	uM	
0.333	water				

Gently but thoroughly vortex the master mix.

Split the master mix into 3 1.5ml centrifuge tubes at room-temperature by adding 9.6µL to each tube. Refreeze the rest of the master mix for later use if desired.

Add the following DNA to each sub-reaction:

Sub-reaction #	DNA1	DNA2
1	Cas DNA	Non-targeting gRNA

2	Cas DNA	PAMSCANR gRNA
3	Cas DNA	Non-targeting gRNA
4	Cas DNA	PAMSCANR gRNA

Gently but thoroughly vortex each sub reaction, then add 5µL of each reaction to the bottom of a V-bottom plate, seal with a cap mat, and incubate at 30˚C. Collect reactions 1 and 2 immediately (time 0) by freezing, and reactions 3 and 4 after the amount of time required for cleavage as determined by the pilot experiment. If the pilot experiment is not possible, set up more reactions and collect a time-series spanning 12hr with both targeting and PAMSCANR gRNA containing spacers collected every 3h.

Sequencing and data analysis

Adding adapters for NGS

1. Make a 1/10 dilution with the TXTL reaction (1uL TXTL reaction, 9µL nuclease-free H2O)

2. Set up a PCR with the components and reaction conditions below:

Component	***Volume (µL)***
DMSO	2
5x Q5 reaction buffer	10
10mM dNTPs	1
10uM RL133	2.5
10uM RL134	2.5
diluted TXTL reaction	1
H2O	30.5
Q5 polymerase	0.5

Temperature (˚C)	***Time (s)***
98	30
98	10
62.9	30
72	7
repeat 25x	
72	120
10	hold

The sequence of the oligos RL133 and RL134 is listed below. The sequence that anneals to the PAM library is listed in lower case.

RL133	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGttcattaaaaattgaattgac attaacct

RL134	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGattcaccaccctgaattgact

3. On Parafilm, add a mixture of 2µL 6x gel loading dye and 10µL of the PCR product

4. Load 10µL into a 1% agarose gel and run with 10µL 100bp ladder at 100V until dye travels ~80% of gel.

5. Image gel. A strong 200bp band should be present.

6. Clean up the PCR product

   a. Vortex the AMPureXP beads
   b. Mix 40µL beads with the PCR product and mix well by pipetting up and down
   c. Incubate mixture at room temperature for 5 minutes
   d. Place PCR tube onto magnetic rack and wait 2 minutes or until supernatant is clear
   e. With the tubes remaining on the magnetic plate, use a pipette to remove supernatant
   f. Wash the beads with 200µL of fresh 80% EtOH to the tube and wait 30 seconds. Remove supernatant and repeat wash step.
   g. After repeating wash step, remove supernatant and let the sample air dry for 10 minutes
   h. Remove PCR tube rack from the magnetic plate, and add 65uL of 10mM Tris-HCl to tube and mix well
   i. Incubate mix for 2 minutes and place the PCR tube back onto the magnetic tube rack and incubate an additional 2 minutes.
   j. Transfer 60uL of the supernatant to a new tube ensuring that no beads are transferred.
   k. Nanodrop the sample. Average concentration should be ~12ng/µL

Adding indices for NGS

7. With the adapters on the library, Illumina indices and binding domains must be attached. Thus, these primers will be different for each sample. Run the second PCR using the components and reaction conditions below.

Indexing primers can be found at here. We used the Nextera indexing primers found on page 12 of the document.

Component	***Volume (uL)***
5x Q5 reaction buffer	10
10mM dNTPs	1
10µM Index1 (i7) read	2.5

Temperature (C)	***Time (s)***
98	30
98	10
67	20

10μM Index2 (i5) read	2.5
purified PCR product	5
H2O	28.5
Q5 polymerase	0.5

72	10
repeat 8x	
72	120
10	hold

8. Clean the second PCR product using the same protocol as in step 6, except for the following changes

   a. Mix 56uL beads with the PCR product
   b. Add 27uL 10mM Tris-HCl and transfer 25uL to a new tube

9. Measure the final sample concentration using a Nanodrop. The concentration should be ~40ng/μL.

10. Run ~100ng of total DNA on a 1% gel (2μL 6x loading dye, 100ng PCR product, up to 10uL H2O) with 10μL 100bp ladder. Run at 100V until dye travels ~80% of gel.

11. Image gel. The band should be at the 300bp marker, and the intensity should be comparable to the band corresponding to the 100ng marker.

12. Prepare the samples appropriately (indicated by chosen sequencing company). Ensure that the company knows that this is a low complexity library and to spike in phage DNA as needed (we've used 15%).

Analyzing the sequencing data

We have written a simple python script to count the representation in each PAM for the 5N PAMSCANR libraries. The script would need to be adapted to other setups, including assaying the 10N library above. We have made the script available in the following git repository:

https://bitbucket.org/csmaxwell/crispr-txtl-pam-counting-script

There are basic instructions included in the repository to use the script.

Information for creating PAM wheels can be found in Leenay et al. 2016:

http://dx.doi.org/10.1016/j.molcel.2016.02.031